Oxalic Acid Production by *Sclerotinia homoeocarpa*: the Causal Agent of Dollar Spot

A Senior Honors Thesis

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Abstract

Fungi in the genus *Sclerotinia* include some of the most devastating plant pathogens known. Oxalic acid is a key pathogenicity factor and its production along with pectolytic cell wall-degrading enzymes by species within this genus is well documented. Dollar spot, caused by *S. homoeocarpa*, is the most prevalent and sprayed for disease of golf course turf. However, nothing is known about acid production or its role in this species. A series of laboratory-based assays were used to determine whether *S. homoeocarpa* produces oxalic acid. Potato dextrose agar (PDA) adjusted to pH 4 and pH 6, with and without bromophenol blue (Bb), was used to assess the growth of and acid production by *S. homoeocarpa* between 5-35 C. When added to PDA, Bb only slightly hindered the growth of *S. homoeocarpa*. Acid production by *S. homoeocapra* on PDA + Bb occurred between 15-30 C at both pHs, but was first observed on media adjusted to pH 6. Maximum acid production occurred between 20-30 C. Acid production by *S. homoeocarpa* was also observed when grown in potato dextrose broth (PDB) at 25 C. High performance liquid chromatography analysis of spent culture broth collected from *S. homoeocrapa* inoculated PDB revealed the presence of oxalic acid.

Introduction

Fungi in the genus *Sclerotinia* include some of the most devastating plant pathogens of many economically important crops. *S. sclerotiorum* has a broad host range and can colonize over 400 species worldwide, with the majority of these species being dicotyledons (Boland and Hall, 1994). It has a very destructive nature and annual losses from the fungus in the United States have exceeded $200 million (Bolton et al, 2006). *S. rolfsii* is another nonspecific pathogen and infects over 500 plant species (Liu et al, 2007). The diseases caused by this fungus are commonly known as southern blight, which occur on both monocotyledons and dicotyledons (Bateman and Beer, 1964). *S. trifoliorum* is the causal agent of Sclerotinia crown and stem rot, a destructive disease of alfalfa and other forage legumes (Reichard et al, 1997), and *S. minor* incites Sclerotinia blight, which results in significant damage to peanut crops (Livingstone et al, 2005).

Dollar spot, caused by *Sclerotinia homoeocarpa*, is the most prevalent and sprayed for disease of golf course turf (Figure 1). The fungus is widespread in the temperate climates of North America, Europe, and Australia and affects most turfgrasses (Viji et al, 2004). Upon infection, tissue necrosis leads to the characteristic dollar spot symptoms that appear as brown, circular spots, roughly 2-5 cm in diameter on close-cut turf and larger and more diffuse on longer-cut turf (Viji et al, 2004). *S. homoeocarpa* survives as resting bodies called stromata or dormant mycelia in plant tissue, thatch, and clippings (Rimelspach and Boehm, 2001). It spreads through mycelial infection of adjacent plants, wind, water, and unintentional transport such as by humans, animals, mowers, and other turf care equipment (Cornell Factsheet, 2007).

Optimal temperatures for disease severity have been previously established between 15-30 ^{o}C (60-85 ^{o}F) and once temperatures exceed 32 ^{o}C (90 ^{o}F) symptoms

lessen (Smiley et al, 2000). The disease thrives with warm, humid days and cool nights. This can be exacerbated by dew, rain, or irrigation, with prolonged leaf wetness serving as a key requirement for the development of disease (Rimelspach and Boehm, 2001). Although areas of turf can be lost if the disease in not controlled, the problem is mainly cosmetic and economic, decreasing the quality and appearance of the turf. Despite its impacts, relatively little is known about the biology, ecology, and epidemiology of the dollar spot fungus. Additionally, nothing is known about acid production or its role in this species.

S. sclerotiorum, *S. rolfsii*, *S. trifoliorum*, and *S. minor* all produce oxalic acid (Maxwell and Lumsden,1970; Bateman and Beer, 1964, Pierson and Rhodes, 1992; Livingstone et al, 2005). The role of oxalic acid as an essential determinant of pathogencity is well documented. In 1964, Bateman and Beer concluded from their study of infected bean hypocotyls that oxalic acid production by *S. rolfsii* in infected tissue functions to create an acidic environment favorable for polygalacturonase activity. Additionally, it ties up calcium in the pectates of the cell wall, allowing for the rapid destruction of pectic substances within the host, leading to the breakdown of plant tissue. In *S. sclerotiorum*, the oxidative bust of the host plant is suppressed by oxalic acid through the inhibition of H_2O_2 production, most likely via the blocking of a signaling step in the oxidase assembly/activation stream in tobacco and soybean cells (Cessna et al, 2000). Guimarães and Stolz showed that oxalate production leads to guard cell dysfunction as a result of *S. sclerotiorum* infection, causing foliar dehydration. Additionally, Sclerotinia-secreted oxalic acid was shown to be an elicitor of programmed cell death in plants and is responsible for induction of apoptotic-like features in the plant

during disease development (Kim et al, 2008). Moreover, *S. sclerotiorum* mutants unable to produce oxalic acid proved to be nonpathogenic on bean plants, while the oxalic acid-producing wild type was pathogenic (Godoy et al, 1990).

The goals of this study were to establish growth curves of *S. homoeocarpa* at varying pHs and temperatures, to determine if *S. homoeocarpa* produces an acid and if so, to identify it and assess the impact of temperature and pH on its production.

Materials and Methods

Two biological replicates of laboratory-based assays were used to carry out this study. First, growth curves were established to assess the optimal incubation times and temperatures for mycelia growth on plate cultures. Additionally, the media was adjusted to pH 4 and pH 6 and bromophenol blue was added to assess acid production (Steadman et al, 1994). Next, broth culture was used to grow the fungus. Culture filtrate extracts were analyzed via high performance liquid chromatography (HPLC) to determine the presence of oxalic acid (Martz et al, 1990).

Growth Curve Determination. Potato dextrose agar (PDA) was adjusted to pH 4 and pH 6 by 85% lactic acid and 1M potassium hydroxide, respectively, with and without bromophenol blue (Bb). The media was inoculated with *S. homoeocarpa* isolate B1 obtained from The Ohio State University, Ohio Turfgrass Foundation Research & Educational Facility and incubated for ten days at five degree increments from 5-35 C. Mycelial growth was determined by measuring diameter growth every 24 hours. These data were subsequently used to calculate the Area Under the *S. homoeocarpa* Growth

Curve (Steadman et al, 1994). ANOVA tests were used to analyze the results for statistical significance.

Acid Production Determination. Acid production was qualitatively documented, on PDA + Bb plates inoculated with *S. homoeocarpa* as evident by a color change in the media from purple to yellow (Steadman et al, 1994). Acid production was ranked on a scale from no production (-) to maximum production (+++) based on the degree of color change observed on the bromophenol blue amended PDA plates (Figure 2 shows the qualitative scale used in this study).

Acid Identification. Potato Dextrose Broth (PDB) adjusted to pH 4 and pH 6, following the aforementioned procedures, was inoculated with *S. homoeocarpa* and incubated at 25 C for 17 days. The pH was recorded every three days. After 17 days, the spent broth was tested for the presence of oxalic acid via HPLC analysis (Figure 4). An oxalic acid (Sigma, St. Louis, MO) standard curve using concentrations from 5 mM to 0.1 mM and samples spiked with 5 mM oxalic acid were used as internal controls (Martz et al, 1990).

Results

S. homoeocapra mycelial growth is most rapid between 15-30 C. 20-25 C are the optimal temperatures. There was no statistical significance in growth between pH 4 and pH 6 or by the addition of Bb (Figure 3).

S. homoeocarpa produces an acid as evident by the acidification of both the agar and broth media. The acid was identified as oxalic acid, which is characteristic of other pathogenic *Sclerotinia* species (Figure 5). Oxalic acid production appears to be closely tied to temperature. It is tightly regulated between 15-30 C with 20-30 C being the temperatures where acidification is greatest (Figure 3). These temperatures correspond to

the established range that dollar spot symptoms are most severe in the field. Oxalic acid production also appears to be pH-dependent, with more alkaline conditions inducing production earlier (Figure 3).

Discussion

This study concludes that oxalic acid is produced by *Sclerotinia homoeocarpa*. This is a significant finding due to the lack of knowledge on this particular species and how it causes disease. Oxalic acid production by other *Sclerotinia* is an integral component of host pathogenesis (Bateman and Beer, 1964). This suggests that oxalic acid may also plays an important role in turfgrass pathogenesis by *S. homoeocarpa*.

Mycelial growth of *S. homoeocarpa* was most rapid between 20-30 ^{o}C. Interestingly, oxalic acid production by *S. homoeocarpa* was also greatest in this same temperature range. This could signify a relationship between quantity of mycelial growth and quantity of oxalic acid production. However, Bateman and Beer claimed that oxalic acid production was not directly related to the total mycelium produced in their study, but rather the carbon source. More importantly, this temperature range corresponds to severe dollar spot symptoms in the field (Smiley et al, 2004). Since maximum acid production is taking place in the same range, this could further the argument that oxalic acid is an important factor involved in the pathogenicity of *S. homoeocarpa* on turfgrass.

The initial pH of the media appeared to influence the rate of oxalic acid production by *S. homoeocarpa*. Acidification of the media was first present at pH 6 in comparison to pH 4 (Figure 3). Maxwell and Lumsden showed that the pH of the growth media is a primary factor in regulating oxalic acid accumulation by *S. sclerotiorum* in a glucose-sodium succinate medium. Also, it appears that *Sclerotinia* act to create a

favorable environment for acid-stable enzyme activity (Bateman and Beer, 1964). In *S. rolfsii* infection of bean hypocotyls, tissue pH dropped from pH 6.0 to pH 4.0, which was optimum for polygalacturonase activity (Bateman and Beer, 1964). This evidence suggests that oxalic acid could also play a role in creating an environment in which cell wall-degrading enzymes secreted by *S. homoeocarpa* are upregulated. These enzymes could lead to rapid destruction of the pectic substances within the host as described by Bateman and Beer. Initial PDA+Bb work with *S. sclerotiorum* and *S. trifoliorum*, following the same procedures as previously mentioned, showed a quicker and more intense acidification of the media in comparison to *S. homoeocarpa* (results not shown). However, *S. sclerotiorum* and *S. trifoliorum* predominantely infect dicotyledons, while *S. homoeocarpa* infects monocotyledons. Monocotyledons contain less pectin in the cell wall in comparison to dicotyledons (Jarvis et al, 1988), so this could be a potential reason if oxalic acid production concentrations are less with *S. homoeocarpa*.

Further studies of *S. homoeocarpa* include a time-course study of the quantification of oxalic acid concentrations in spent broth at pH 4 and pH 6 and a time-course study of the quantification of secreted proteins by *S. homoeocarpa* in spent broth.

Works Cited

Boland, G. J., and R. Hall. (1994). "Index of Plant Host of *Sclerotinia sclerotiorum*." Canadian Journal of Plant Pathology, 16: 93-108.

Bolton, Melvin D., Bart P. H. J. Thomma, and Berlin D. Nelson. (2006). "*Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen." Molecular Plant Pathology, 7: 1-16.

Cessna, Stephen A., Valerie E. Sears, Martin B. Dickman, and Phillip S Low. (2000). "Oxalic Acid, a Pathogenicity Factor for *Sclerotinia sclerotiorum*, Suppresses the Oxidative Burst of the Host Plant." The Plant Cell, 12: 2191-2199.

"Dollar Spot of Turfrass, *Sclerotinia homoeocarpa*: Factsheet." (2007). The Plant Disease Diagnostic Clinic. Cornell University. <http://plantclinic.cornell.edu/factsheets/dollar_spot/dollar_spot.htm>.

Godoy, G., Steadman, JR., Dickman, M.B., Dam, R. (1990). "Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*." Physiological and Molecular Plant Pathology, 37: 179–191.

Jarvis, Michael C., Wilson Forsyth, and Henry J. Duncan. (1988). "A Survey of the Pectic Content of Nonlignified Monocots Cell Walls." Plant Physiology, 88: 309-314.

Kim, Kyoung Su, Ji-Young Min, and Martin B. Dickman. (2008). "Oxalic Acid Is an Elicitor of Plant Programmed Cell Death during *Sclerotinia sclerotiorum* Disease Development." Molecular Plant-Microbe Interactions, 21: 605-612.

Liu, Bo, Marcia L. Gumpertz , Shuijin Hu , and Jean Beagle Ristaino. (2007). "Long-term effects of organic and synthetic soil fertility amendments on soil microbial communities and the development of southern blight." Soil Biology and Biochemistry, 39: 2302-2316.

Livingstone, D. Malcolm, Jaime L. Hampton, Patrick M. Phipps, and Elizabeth A. Grabau. (2005). "Enhancing Resistance to *Sclerotinia minor* in Peanut by Expressing a Barley Oxalate Oxidase Gene." Plant Physiology, 137: 1354–1362.

Martz, F.A., M. F. Weiss, and R. L. Belyea. (1990). "Determination of Oxalate in Forage by Reverse-Phase High Pressure Liquid Chromatography." Journal of Dairy Science, 73: 474-479.

Maxwell, Douglas P. and R. D. Lumsden. (1970). "Oxalic Acid Production by *Sclerotinia sclerotiorum* in Infected Bean and in Culture." Phytopathology, 60: 1395-1398.

Reichard, S. L., R. M. Sulc, and L. H. Rhodes. (1997). "Growth and reproduction of *Sclerotinia trifoliorum* as influenced by herbicides." Mycologia, 89: 82-88.

Rimelspach, Joseph W. and Michael J. Boehm. (2001). "Dollar Spot on Turfgrass." Ohio State University Extension Fact Sheet. The Ohio State University. <http://ohioline.osu.edu/hyg-fact/3000/3075.html>.

Smiley, Richard W., Peter H. Dernoeden, and Bruce B. Clarke. Compendium of Turfgrass Diseases. Second Edition. APS Press. 2000.

Steadman, J.R., J. Marcinkowska, and S. Rutledge. (1994). "A semi-selective medium for isolation of *Sclerotinia sclerotiorum*." Canadian Journal of Plant Pathology, 16: 68-70.

Viji, G., Uddin, W., O'Neill, N. R., Mischke, S., and Saunders, J. A. (2004). "Genetic diversity of *Sclerotinia homoeocarpa* isolates from turfgrasses from various regions in North America." Plant Disease, 88:1269-1276.

Figure 1:
S. homoecapra growth on PDA (top), growth and infection on individual turfgrass blade (middle), and widespread infection known as dollar spot (bottom). Pictures courtesy of Dr. Michael Boehm.

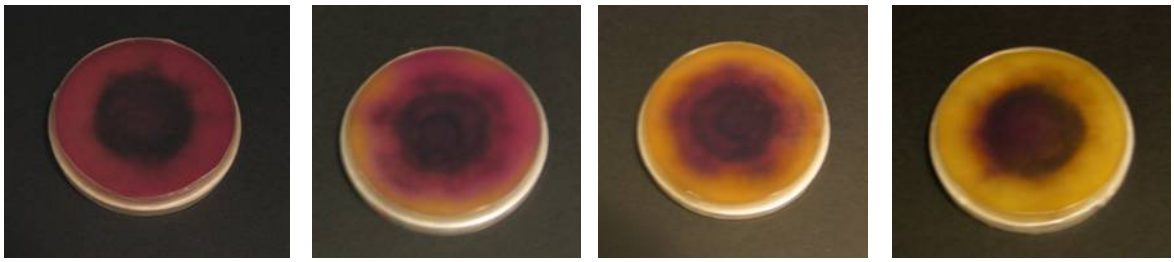


Figure 2:
Qualitative scale of acid production by *S. homoeocapra* on bromophenol blue amended PDA. Acidification of the media is seen as a color change from purple to yellow. The scale ranked from no production (-) to maximum production (+++), based on the degree of color change.

Area Under *S. homoeocarpa* Growth Curve with Acid Production Overlay

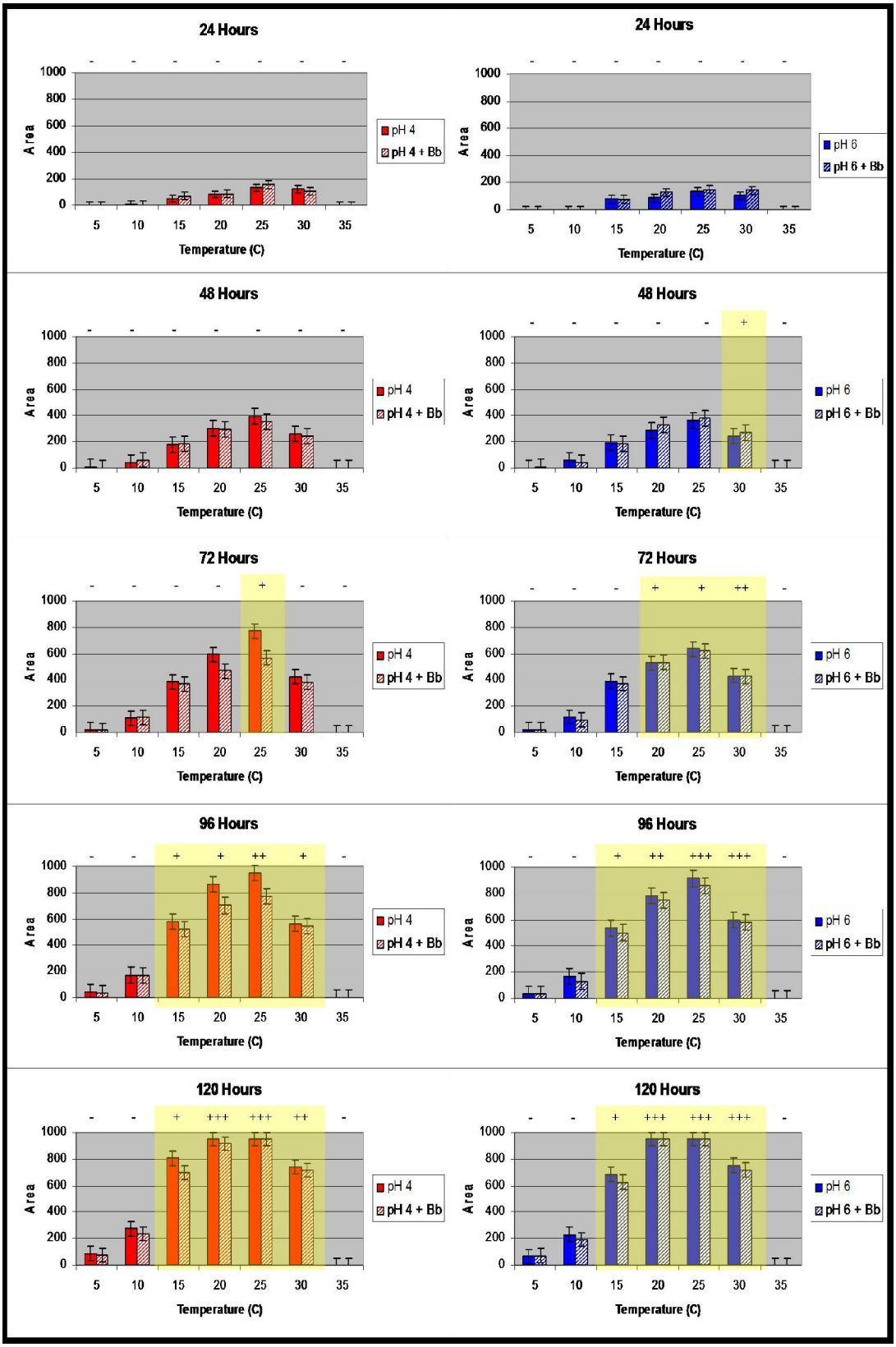
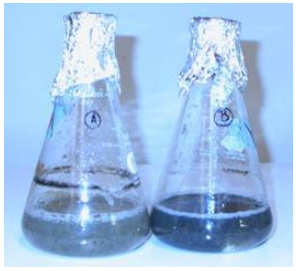
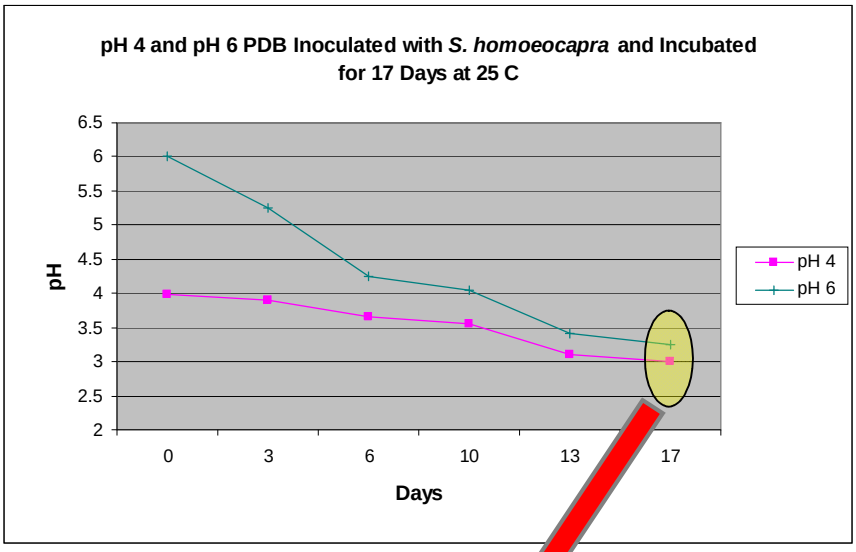
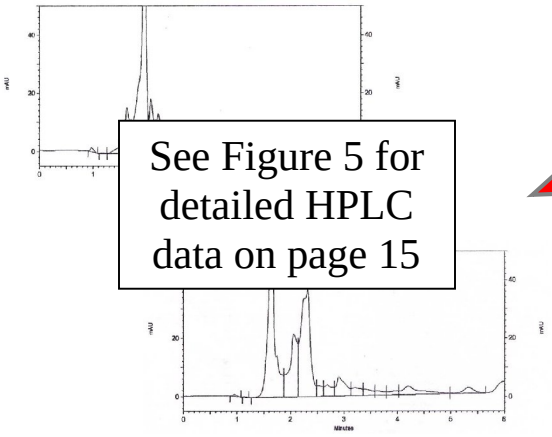


Figure 3:
Area Under *S. homoeocarpa* Growth Curve for temperatures 5-35 C after the designated amount of time with acid production overlay. This graph shows the highest growth rate between 15-30 C with 20-30 C proving optimal. The earliest and highest qualitative acid production occurred at pH 6 in comparison to pH 4. Additionally, acid production is only present between 15-30 C.







See Figure 5 for detailed HPLC data on page 15

Figure 4:
Acid identification via HPLC analysis of spent broth culture after 17 days incubation. This figure shows the fungus growth in broth culture (top). Every three day, the pH was tested and recorded for 17 days (middle right), after 17 days, the spent broth was analyzed by HPLC (bottom right). Detailed HPLC results are shown in Figure 5.

HPLC analysis:

Standard:
PDB (no oxalic acid present)

Standard:
PDB + 1 mM oxalic acid

Trial:
PDB + *S.homoeocarapa*
pH 6.0
(oxalic acid present)

Trial:
PDB + *S. homoeocarpa*
pH 4.0
(oxalic acid present)

Trial:
PDB + *S. homoeocarpa*
pH 4.0 + oxalic acid spike
(oxalic acid production confirmed)

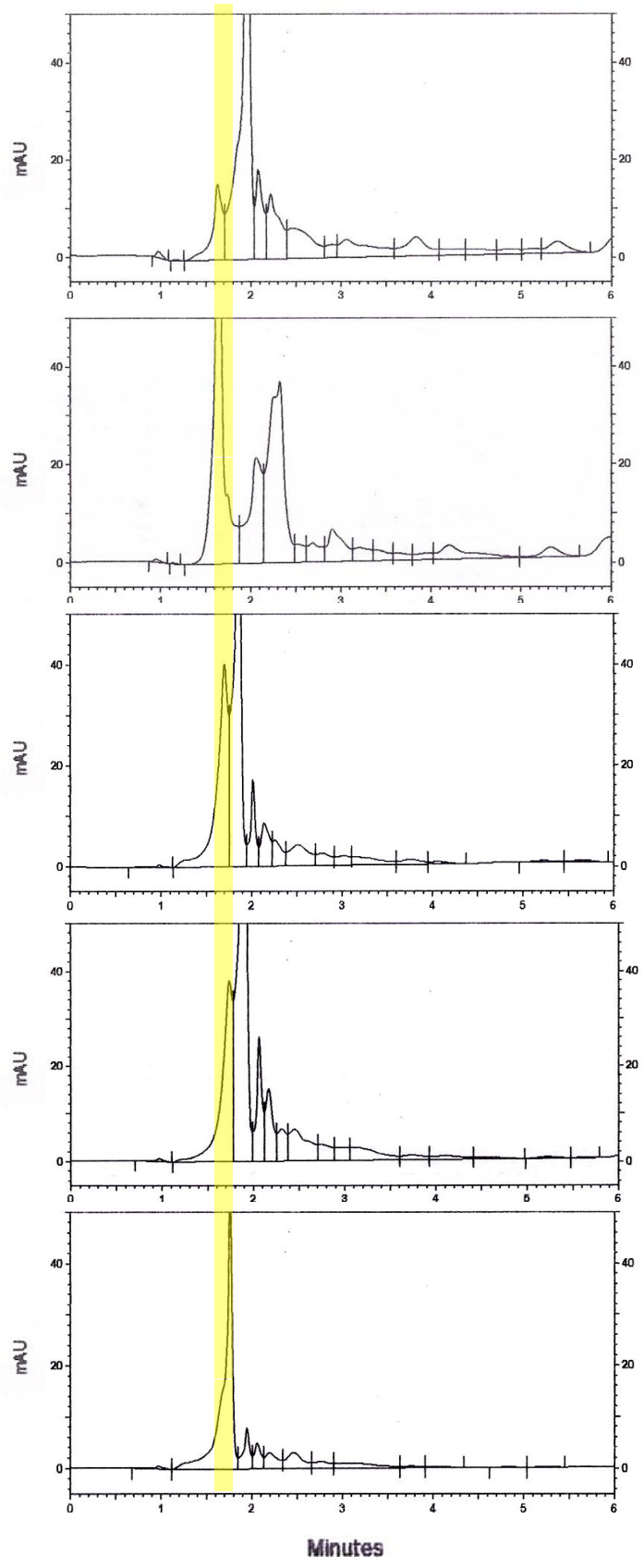


Figure 5:
The results showed the presence of oxalic acid. The retention time established via oxalic acid standards was 1.6-1.7 minutes. All samples corresponded to this time, shown by the yellow band.